Stress-induced anxiety is dependent upon caspase-1 processing of active IL-1β in brain macrophages

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Abstract

The murine repeated social defeat (RSD) model reveals that neuroimmune signaling is implicated in the development of stress-induced anxiety-like behavior. Work from our lab showed that the anxiety-like behavior exhibited following RSD is associated with microglial activation, increased recruitment of brain-macrophages, and increased IL-1β expression in the brain. Moreover, this neuroinflammatory process was dependent upon IL-1 receptor 1 in the CNS. To determine the source of cytokine production following stress, NanoString mRNA analysis was conducted on FACS-sorted microglia and brain-macrophages. This revealed that brain macrophages rather than resident microglia are the primary source of increased IL-1β mRNA production in the brain following repeated social defeat. To address the functional role of IL-1β in the development of anxiety-like behavior, caspase-1 knockout (KO) mice were used. Caspase-1 is required for the cleavage of pro-IL-1β into its active form. Results show that caspase-1 KO mice did not develop anxiety-like behavior following RSD. Furthermore, our data indicates that caspase-1 KO mice do not exhibit increased microglial activation as characterized by Iba-1 immunolabeling. However, caspase-1 KO mice still had increased peripheral macrophage trafficking to the brain and increased IL-1β mRNA expression in the brain. We interpret these results to indicate that the development of anxiety-like behavior is dependent on functional IL-1β released by brain macrophages that is cleaved by caspase-1.

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I would like to first thank Dr. Jonathan Godbout, my research advisor, for his mentorship and support throughout my undergraduate research career at The Ohio State University. His assistance over the past three years has provided me a foundation in biomedical research that will be invaluable in my future in the medical field. In addition, I also wish to express my gratitude to Dr. John Sheridan, both as a member of my committee and a mentor as well. Furthermore, I would like to especially thank my graduate student mentor of Dr. Godbout and Dr. Sheridan, Dan McKim. His dedication and patience with me as a student was essential in my development as an undergraduate researcher, and this project is the culmination of his teaching and trust in me as a mentee.

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CHAPTER 1

INTRODUCTION

Psychological stress is described as the perceived stress that influences an individual's ability to process life events and to cope with them (Godbout and Glaser, 2006). Exposure to psychological stress has been shown to have a profound impact on immune system regulation and overall health of an individual (Stark et al., 2011). Acute stress can have beneficial effects on behavior, as exhibited by the "fight-or-flight" fear response. This response activates the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS), allowing for an organism to react to threats accordingly for survival (Godbout and Glaser, 2006). Chronic psychological stress, however, has been shown to have detrimental effects on an organism, often leading to the development of mental health issues such as depression and anxiety (Kiecolt-Glaser and Glaser, 2002; Miller et al. 2009). Prolonged exposure to stress similarly activates the HPA and SNS pathways, leading to the release of catecholamines, glucocorticoids, and pro-inflammatory cytokines (Wohleb et al., 2011). This stress-induced neuroinflammation has a profound negative impact on behavior, mood, and immunity in both human and murine models (Kinsey et al., 2007; Stark et al., 2011).

In one clinically relevant murine model, repeated social defeat (RSD), depression and anxiety-like behavior following chronic stress are correlated with increased neuroinflammation and neuroplasticity due to stress-induced immune dysfunction (Wohleb et al., 2011; Ramirez et al., 2015). Moreover, prolonged activation of the SNS with RSD enhances myelopoeisis to produce immature monocytes ($CD11b^{+}$, $Ly6C^{hi}$)

from the bone marrow and release them into circulation (Sunderkötter et al. 2004, Yona 2010, Powell et al. 2013). These myeloid cells show a decreased sensitivity to glucocorticoid anti-inflammatory feedback (Stark et al. 2011). Furthermore, the glucocorticoid-resistant myeloid cells released from the bone marrow following RSD are more inflammatory, as demonstrated by a marked increase in production of various inflammatory cytokines following lipopolysaccharide (LPS) challenge, such as IL-1β, IL-6, CCL2 and TNF-α (Stark et al. 2001, Avitsur et al. 2003, Bailey et al. 2007). Past work from our lab has shown that these macrophages readily traffic to the brain and induce microglial activation (Wohleb et al., 2011). The accumulation of peripheral macrophages in the brain is associated with the development of anxiety-like behavior and the production of pro-inflammatory cytokines (Wohleb et al., 2013). Moreover, past studies using chemokine receptor-deficient murine models has shown to lead to the prevention of anxiety-like behavior (Wohleb et al., 2013). This is of particular interest because stress-induced anxiety and depression disorders are associated with neuroinflammation (Wohleb et al., 2012).

Previous work by Wohleb et al. indicated that IL-1β could play an integral role in the development of anxiety-like behavior following repeated social defeat (2011). In a study done with IL1-R1 $^{-/-}$ C57BL/6 mice, they found that the deficiency of IL1-R1 was sufficient in preventing the development of anxiety-like behavior (Wohleb et al., 2011). The role of IL1-R1 was further studied using an endothelial-specific knockdown of IL1-R1, which also prevented the development of anxiety following stress (Wohleb et al., 2014, *J Neurosci*). However, the role of IL-1β in the development of anxiety-like behavior still is unclear. Several cell types may be involved in the production of IL-1β

following exposure to psychological stress, including brain macrophages, resident microglia, and endothelial cells (Allan et al., 2005). It is known that increased IL-1β expression in the brain following stress is a major contributor to many of the biological mechanisms that underlie the development of anxiety (Koo and Duman, 2008, Goshen and Yirmiya, 2009). In addition, IL-1β signaling has been implicated as a stimulus for SNS activation, a major component of the immune response to psychological stress (Black, 2002; Murakami et al., 2002). Thus, understanding the role of IL-1β in the response to psychosocial stress could provide potential therapeutic targets for treating stress-induced behavior disorders.

One way to target IL-1β production is through the enzyme caspase-1. Caspase-1 is an intracellular protein that is responsible for cleaving the precursor forms of IL-1β and IL-18 into their bioactive forms (Vanaja et al., 2015). This process is driven by the inflammasome complex, which is activated when the innate immune system is placed under cellular stress (i.e. pathogen, LPS, etc.) via toll-like receptor-4 (TLR4) (Figure 1; Choi and Ryter, 2014). This is important because exposure to RSD is associated with increased TLR4 activation in peripheral macrophage and resident microglia (Bailey et al., 2007; Wohleb et al., 2011). The activation of TLR4 leads to NF-κB signaling within the cell, causing the production of pro-IL-1β and pro-IL-18 and activating NLRP3, which mediates the recruitment and activation of caspase-1 within the cell from pro-caspase-1 (Martinon et al., 2009; Choi and Ryter, 2014). Caspase-1 then proteolytically cleaves pro-IL-1β and pro-IL-18 into their bioactive forms IL-1β and IL-18, which is then released by the innate immune cells (Martinon et al., 2009). Therefore by using caspase-1 deficient mice, IL-1β production should be prevented by affecting the post

translational modification of pro-IL-1β without affecting the activation of the innate immune system following RSD.

The primary objective of this study was to assess the role of IL-1β in the development of anxiety-like behavior. To address this, inflammatory profile analysis of brain macrophages and microglia were determined to assess mRNA expression of each cell type. Secondly, we used caspase-1 deficient mice as a way to analyze the behavior and physiological effects of IL-1β deficiency during stress. We hypothesize that ablating IL-1β production via caspase-1 deficiency will prevent the development of anxiety-like behavior following repeated social defeat.

CHAPTER 2

METHODOLOGY

2.1 Mice

C57BL/6 mice (6-8 weeks old) and CD1 mice (1 year, retired breeders) were obtained from Charles River Breeding Laboratories and given 7 to 10 d to acclimate to their surrounding prior to experimentation. Caspase-$1^{-/-}$ mice on a C57BL/6 background were purchased from the Jackson Laboratory. C57BL/6 mice were housed in a cohort of three and CD-1 mice were singly housed in 11.5 x 7.5 x 6 inch polypropylene cages. The rooms were maintained at 21^{oC} and kept in a 12:12 h light:dark cycle with *ad libitum* access to food and water. Mice were randomly selected for each treatment group. All procedures were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Ohio State University Institutional

Laboratory Animal Care and Use Committee.

2.2 Repeated Social Defeat

Cages of three C57BL/6 experimental mice were randomly assigned to either control (CON) or repeated social defeat (RSD) conditions. CON mice were left undisturbed in their cage throughout the entirety of the experiment in their home cage until sacrifice. In the RSD condition, an intruder CD-1 mouse (12 months old) was placed in a cage with the three resident C57BL/6 mice (6 to 8 weeks old) for two hours between 17:00 and 19:00 for 6 consecutive nights. Each night submissive behavior, such as crouching and fleeing, was observed. If the CD-1 intruder did not initiate an attack within the first 5 to 10 min or was attacked by a resident mouse, a new intruder was introduced. At the end of the 2 h cycle, the intruder was removed and the residents were left undisturbed until the next evening. A new intruder mouse was used for each cage for each cycle of stress. Mice that were injured or dying were removed from the study. Mice from CON and RSD conditions were then sacrificed 14 h following the last cycle of stress for experimental analysis.

2.3 Anxiety-Like Behavior

Anxiety-like behavior was assessed using the open field behavior test. The open field apparatus consisted of a 30 x 30 x 25 cm Plexiglas box with a solid floor and lighting overhead. A grid was drawn on the floor to divide it into 36 squares. The center of the apparatus is defined as the space three squares away from two walls (total of 9 squares). The C57BL/6 mice were placed in the corner of the apparatus 12 h after the

last cycle of stress and given 5 min to roam freely. Locomotor activity was recorded using an automated system (AccuScan Instruments) and analyzed using VersaMap software. Mice that express anxiety-like behavior tend to spend less time in the center of the apparatus, take longer to enter the center for the first time (latency to enter the center), and enter the center fewer times (number of entries).

2.4 Isolation of Brain CD11b^{+} Cells

Brains were collected from experimental mice 14 h after the last cycle of stress. Brains were homogenized in PBS (7.4 pH) by passing through a 70 μm nylon cell strainer. The resulting homogenates were then centrifuged at 900 g for 6 min. Supernatants were discarded and the cell pellets were resuspended in a discontinuous isotonic Percoll (GE Health) gradient (70%, 50%, 35%, 0%). The gradient was centrifuged at 2,000 g for 20 min, and monocytes (microglia and macrophages) were collected at the 50% to 70% layer. Cells were washed and resuspended in PBS and then analyzed using Flow Cytometry.

2.5 Microglia Staining and Flow Cytometry

Flow cytometry was used to quantify surface protein expression to determine levels of brain microglia (CD11b^{+}, CD45^{lo}) and peripheral macrophages (CD11b^{+}, CD45^{hi}) in the brain following RSD. Monocyte Fc receptors were blocked with an anti-CD16/CD32 antibody (eBioscience). Cells were then washed and incubated with appropriate conjugated antibodies CD45, CD11b, CD86, and Ly6C (eBiosceince) for 45 minutes. Cells were then washed and resuspended in FACS buffer (2% FBS in HBSS

with 1 mg/ml sodium azide) for analysis. Non-specific binding was assessed using non-specific, isotype-matched antibodies. Antigen expression was determined using a Becton-Dickinson FACSCaliber four-color cytometer. Ten thousand events were recorded for each sample and isotype-matched conjugate. Data were analyzed using FlowJo software (Tree Star) and gating for each antibody was determined based on nonspecific binding of appropriate negative isotype stained controls.

2.6 Fluorescence-activated Cell Sorting of Monocytes

To specifically gate for microglia, fluorescence activated cell sorting (FACS) was used. FACS was performed using a a BD FACSAria^{TM} IIu (Becton Dickinson, Franklin Lakes, NJ) with a 488 and 633 nm lasers for excitation of fluorophores. Microglia ($CD11b^{+}$, $CD45^{lo}$) and macrophages ($CD11b^{+}$, $CD45^{hi}$) were specifically targeted and gated using specific cell surface markers. Flow rate for cell sorting was initially set at "low" prior to each sample (0.008 mL min^{-1}). Data analysis was conduced using FACS Diva v. 6.1.3 (Becton Dickinson). All samples were collected and saved for NanoString analysis.

2.7 NanoString assay mRNA Analysis

mRNA expression of FAC-sorted microglia and macrophages was assessed via the NanoString nCounter system (NanoString Technologies, Seattle, WA). The NanoString nCounter system enables multiplexed direct digital counting of mRNA molecules. The raw count data from samples were normalized using the NanoStringNorm R package. Then, data were background-corrected by subtracting the

mean of the negative controls included and then normalized by using the geometric mean of the 10 probes with the lowest coefficients of variation. It is important to note that the probes had to be detectable in over 60% of the samples to be considered in analysis. Probes with expression levels below the level of detection (LOD) were regarding as not detected (n.d.). All values were expressed as relative count expression.

2.8 Morphological Analysis of Microglia

To analyze microglia morphology, brain samples were stained for Iba-1. Mice were deeply anesthetized and perfused transcardially with PBS (pH 7.4 with EDTA) and 4% formaldehyde. Brains were postfixed in 4% paraformaldehyde for 24 h then incubated in 20% sucrose for an additional 48 h. Brains were then frozen with isopentane (-80^{o}C) and sectioned (25 μm) using a Microm HM550 crysotat (Mikron Instruments). Brain regions were identified with the stereotaxic mouse brain atlas. Sections were initially washed in PBS and then incubated with a rabbit anti-mouse Iba-1 antibody (1:1000, Wako Chemicals). Sections were then washed in PBS and incubated with a fluorochrome-conjugated secondary antibody (1:500, Alexa Fluor 594). Brains sections were counter-stained with a DNA stain, 4',6-diamidino-2-phenylindole (DAPI). Sections were then cover slipped with Permount (Fischer-Scientific) and stored at -20^{o}C. All sections were analyzed using confocal microscopy. To quantify the percent area of microglia morphology, digital image analysis (DIA) was performed using Image J. Images from each brain area (hippocampus, medial amygdala, and paraventricular nucleus) were taken at 20x magnification. Proportional area was recorded as the

average percent area in the positive threshold for all pictures.

2.9 RNA Isolation and real-time PCR

RNA was isolated from the Percoll-isolated cells using the PrepEase RNA Spin Kit (Affymetrix). cDNA was generated from the resulting RNA using an RT-RETROscript kit (Ambion). Taqman quanitative PCR was performed using the Applied Biosystems Assay-on-Demand Gene Expression Protocol. Briefly, cDNA was amplified by real time PCR for target cDNA (e.g. IL-1β, TNF-α, etc.) and a reference cDNA (glyceraldehyde-3-phosphate) using an oligonucleotide probe with a 5' fluorescent reporter dye (6-FAM) and a 3' quencher dye (TAMRA). Fluorescence was quantified using an ABI PRISM 7300 sequence-detection system (Applied Biosystems). Data were analyzed using the comparative threshold cycle method and results were expressed as fold difference.

2.10 Calculations and Statistical Significance

Data were subjected to Shapiro-Wilk test using Statistical Analysis Systems (SAS) software to ensure a normal distribution. Observations greater than 3 standard deviations from the mean were considered outliers and were excluded from further analysis. To determine main effects and interactions between main factors, data were analyzed using one (stress, Casp1KO) or two-way (stress x Casp1KO) ANOVA using the general linear model procedures of SAS. All data are expressed as treatment means ± SEM.

CHAPTER 3

RESULTS

3.1 Peripheral macrophages exhibit exaggerated IL-1β expression in the brain following repeated social defeat

Previous work in our laboratory showed that the development of anxiety-like behavior following repeated social defeat was mediated by peripheral macrophage recruitment to the CNS (Wohleb et al., 2013). Moreover, this recruitment of monocytes was correlated with increased mRNA expression of inflammatory cytokines, most notably IL-1β (Wohleb et al., 2014, *Biol. Psychiatry*). To understand the role of the peripheral macrophages in the neuroinflammatory signaling pathway, a NanoString analysis was conducted on macrophages and microglia following repeated social defeat to determine the respective inflammatory profiles.

Figures 2A-2D show four cell-type specific RNA controls used to verify the purity of microglia and macrophages collected after FAC-sorting. CX3CR1 (Fig 2A) is a chemokine receptor highly expressed by microglia (Harrison et al., 1998; Mildner et al., 2007). Fig 2A shows that the microglia isolated significantly express more CX3CR1 than microglia ($p < 0.05$). LysM (Fig 2B) and Ly6C (Fig 2C) are two macrophage-specific markers that can be used to distinguish them from microglia (Powell et al., 2013; Vanella et al., 2014). Our results show that macrophages express LysM ($p < 0.05$) and Ly6C ($p < 0.05$) at significantly higher levels than microglia. Finally, CD45 expression was also used to differentiate microglia ($CD45^{low}$) and macrophages ($CD45^{hi}$) (Nair et al., 2007). Figure 2D shows that the macrophages isolated

significantly express higher levels of CD45 than microglia ($p < 0.05$).

Figure 2E shows that macrophages ($CD11b^{+}$, $CD45^{hi}$) have significantly higher expression of IL-1β at baseline than microglia ($CD11b^{+}$, $CD45^{low}$) ($p < 0.05$). Furthermore, IL-1β expression by macrophages was significantly increased by stress as compared to control conditions ($p < 0.05$). However, stress did not affect the expression of microglia IL-1β. Analysis of IL1-R1 mRNA expression showed converse results. Microglia had a significantly higher expression of IL1-R1 at baseline when compared to macrophages (Fig. 2E) ($p < 0.05$). Moreover, the expression of IL1-R1 in microglia was significantly increased following stress ($p < 0.05$), but no significant increase of IL1-R1 in macrophages was seen after exposure to repeated social defeat. These results indicate that increased IL-1β expression following stress is driven by peripheral macrophages.

3.2 Caspase-1 deficiency prevented stress-induced anxiety-like behavior

Previous work in our lab showed that the development of anxiety-like behavior is dependent upon the recruitment of peripheral myeloid cells ($CD11b^{+}$, $CD45^{hi}$) to the brain (Wohleb et al., 2014, *J Neurosci*). To test the role of IL-1β in the development of anxiety-like behavior, the effect of caspase-1 deficiency on was analyzed. For this experiment, wild-type (WT) and caspase-1 deficient (Casp1KO) mice were subjected to repeated social defeat, and anxiety-like behavior was examined using the open field test 12 h after the last cycle of stress.

Figure 3B shows that WT mice had a decreased amount of time in the center of the apparatus ($p < 0.05$) after exposure to RSD. Figure 3C shows that WT mice had an

increased latency to enter the center ($p < 0.05$) after exposure to RSD as compared to CON mice. Mice subjected to RSD had a decreased total number of entries in the center of the apparatus following stress than did their CON counterparts ($p < 0.05$) (Fig 3D). Moreover, caspase-1 deficiency reduced anxiety-like behavior, as shown by increased time spent in the center ($p < 0.05$), decreased latency to enter the center ($p < 0.05$), and increased number of entries into the center of the apparatus ($p < 0.05$) as compared to WT mice exposed to stress (Fig 3B-D). These results indicate that caspase-1 deficiency returned anxiety-like behavior back to baseline conditions.

3.3 Activated microglial morphology caused by repeated social defeat was prevented by caspase-1 deficiency

Previous work by Wohleb et al. showed that the development of anxiety-like behavior following repeated social defeat was associated with changes in microglia morphology (2011). Therefore, changes in microglia morphology using Iba-1 expression was determined in the medial amygdala (MeA), dentate gyrus of the hippocampus (HPC), and paraventricular nucleus (PVN) of caspase-1 deficient mice. Iba-1 is a surface protein expressed specifically in microglia and is used to differentiate morphology of microglia (Wohleb et al., 2011). Microglial activation is associated with a higher microglia percent area (Wohleb et al., 2011). Figure 4A displays representative samples from all four brain regions from caspase-1 deficient mice in CON and RSD conditions. In all three brain regions analyzed, no significant difference was observed between caspase-1 samples in CON or RSD conditions. Repeated social defeat did not

decrease percent area of microglia in the medial amygdala (Fig 4B), hippocampus (Fig 4C), or paraventricular nucleus (Fig 4D). One potential issue of these results was the lack of control samples able to be analyzed due to tissue damage during preparation. Altogether, this data indicates that caspase-1 deficiency prevents morphological changes of brain microglia following repeated social defeat.

3.4 Caspase-1 deficiency did not prevent macrophage trafficking to the brain nor increased IL-1β mRNA expression in microglia following RSD

Previous work showed that the development of anxiety-like behavior following repeated social defeat is dependent upon the recruitment of peripheral macrophages ($CD11b^{+}$, $CD45^{hi}$) to the brain (Wohleb et al., 2013). Moreover, this is associated with an increase in the production of inflammatory cytokines (i.e. IL-1β, TNF-α, CD14, etc.) by resident microglia (Wohleb et al., 2014, *Biol. Psychiatry*). To test for macrophage trafficking, brains were collected from caspase-1 deficient and wild-type mice 14 h after the last cycle of stress and analyzed via flow cytometry. Figure 5A shows representative flow bivariate plots of CD11b/CD45 labeling in Percoll isolated microglia and macrophages for each experimental condition. Figure 5B shows that stress significantly increased the percent of peripheral macrophages in the brain in both WT ($p < 0.05$) and Casp1KO mice ($p < 0.05$) as compared to CON mice. To test for IL-1β mRNA expression in microglia following stress, rt-PCR analysis of brain microglia was done (Fig 5C). Figure 5C shows the relative mRNA expression of IL-1β in brain microglia from WT and Casp1KO mice in both test conditions. Stress significantly increased the expression of IL-1β mRNA in WT ($p < 0.05$) and Casp1KO ($p < 0.05$)

mice as compared to CON conditions. These results indicate that caspase-1 deficiency had no effect on the recruitment of peripheral myeloid cells after exposure to repeated social defeat. Furthermore, caspase-1 deficiency did not affect the expression of IL-1β mRNA in brain microglia following stress.

CHAPTER 4

DISCUSSION

Our results indicate that peripheral macrophage processing and expression of IL-1β is essential for the development of anxiety-like behavior. Based on our results, peripheral macrophages express IL-1β more highly than resident microglia before and after exposure to stress. Moreover, stress caused a significant increase in the expression of IL-1β in macrophages, but stress had no effect on the expression of IL-1β in microglia. It is important to note that microglia had a significantly higher expression of IL-1R1, a major receptor for IL-1β, than did macrophages. Furthermore, microglial expression of IL1-R1 was significantly increased following stress, but no effect of stress on macrophage IL1-R1 expression was seen. These results suggest that macrophages have a crucial role in the production of IL-1β after exposure to repeated social defeat and that interaction with IL1-R1 on resident microglia could provide an important biochemical connection between the macrophages and microglia following exposure to psychological stress.

The next experiment showed that caspase-1 deficiency prevented the development of anxiety-like behavior following repeated social defeat. It is important to

also note that caspase-1 deficiency did not prevent the accumulation of peripheral myeloid cells from the bone marrow to the brain. Thus, these results indicate that the ability for macrophages to express IL-1β is necessary for the development of anxiety-like behavior. Moreover, our results also indicated that caspase-1 deficiency prevented stress-induced morphological changes of microglia after exposure to stress. Work by Wohleb et al. showed that microglia exhibit hypertrophy following stress and adopt a more "de-ramified" morphology (2011). These results indicate that microglia from caspase-1 deficient mice do not have an activated morphology in multiple brain areas following stress. These results suggest that IL-1β production by brain macrophages via caspase-1 activity is necessary to activate brain microglia during stress.

Finally, the expression of IL-1β mRNA by brain microglia was not affected by caspase-1 deficiency. Past work in our laboratory has shown that the accumulation of peripheral macrophages in the brain leads to increased pro-inflammatory cytokine production by the resident microglia (Wohleb et al., 2014, *Biol. Psychiatry*). Here we extend the past findings by showing increased IL-1β mRNA expression following the trafficking of peripheral monocytes to the brain despite the prevention of anxiety-like behavior. These results suggest that the recruitment of macrophages to the brain increases the expression of pro-inflammatory cytokines mRNA (e.g. IL-1β) independent of caspase-1 activity.

It is important to note that while our results suggest that caspase-1 deficiency affects peripheral macrophage activity, other interpretations for the role of IL-1β in the development of anxiety-like behavior exist. One alternate interpretation is that the recruitment of macrophages to the brain is dependent upon IL-1β expression by

microglia, which leads to the exacerbation of neuroinflammation and anxiety-like behavior. However, the results of this study indicate that brain macrophage expression of IL-1β is necessary for the development of anxiety-like behavior given the recruitment of peripheral macrophages to the brain without the development of anxiety-like behavior.

Overall, these results indicate that caspase-1 processing of IL-1β in brain macrophages is necessary for the development of anxiety-like behavior. It is important to note that this intervention prevented the development of anxiety-like behavior following RSD despite the recruitment of peripheral myeloid cells to the brain. Previous work from our lab before has shown that the recruitment of macrophages is associated with the development of anxiety-like behavior, and the past interventions studied have aimed to prevent anxiety-like behavior by inhibiting the accumulation of macrophages in the brain (Wohleb et al., 2011; Wohleb et al., 2014, *J Neurosci*). The results of this study are relevant because they indicate a novel mechanism related to the development of anxiety-like behavior following RSD. Furthermore, the findings from this study could give further insight into the pathophysiology behind persistent anxiety disorders, such as post-traumatic stress disorder (PTSD), and provide potential therapeutic targets for future treatments of these disorders.

Figure 1

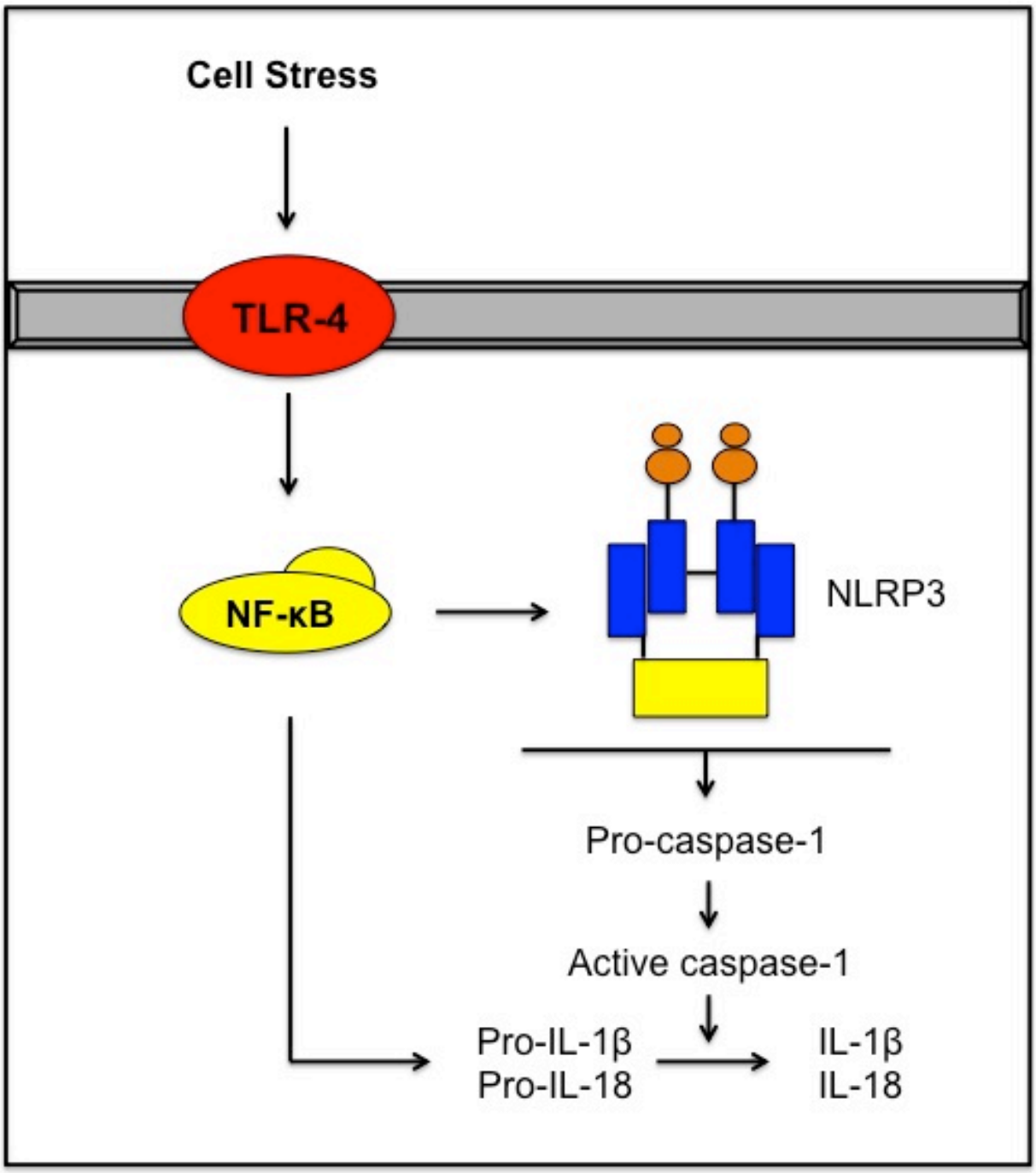
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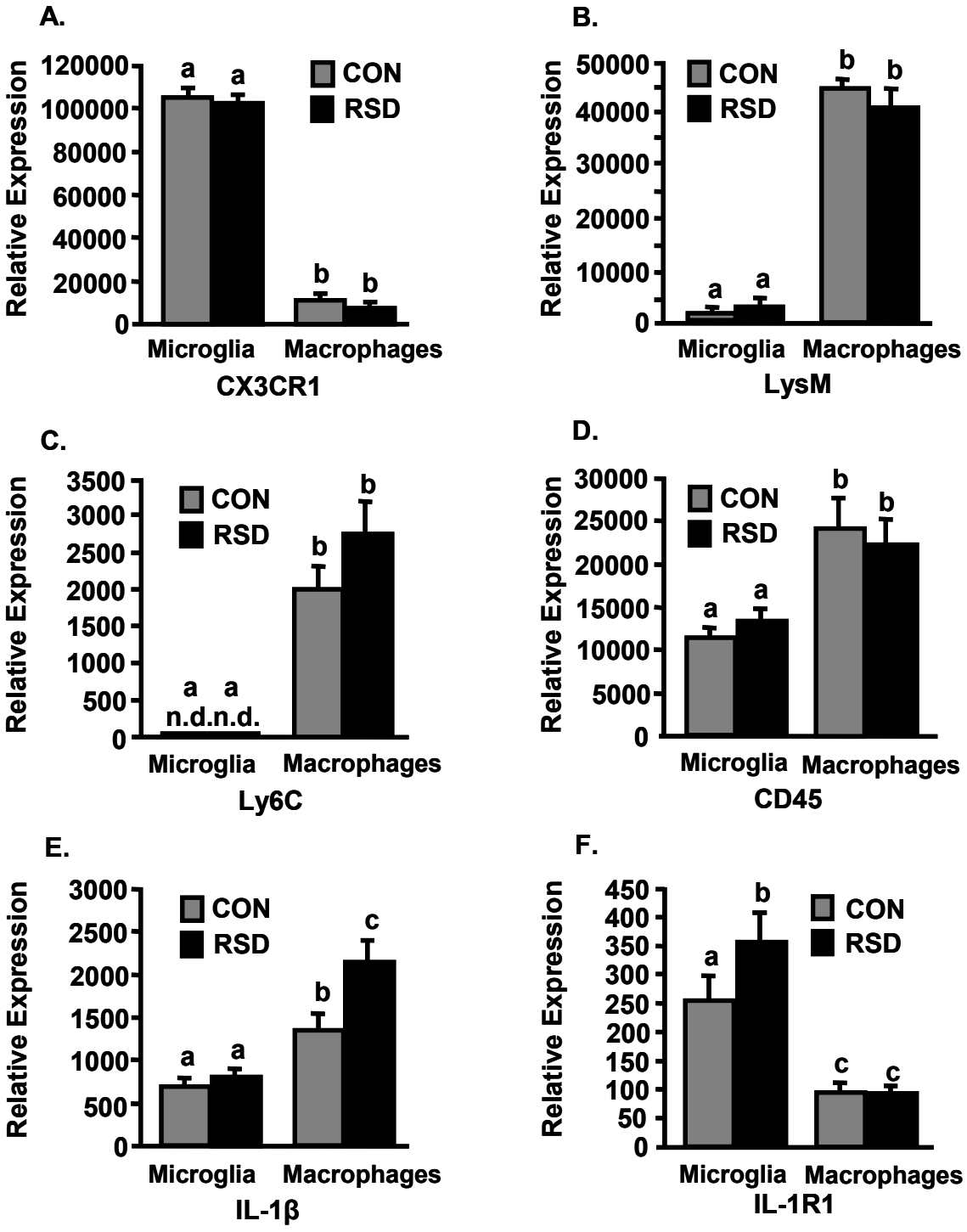
Figure 2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Microglia
Macrophages
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Relative Expression
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F.
Relative Expression
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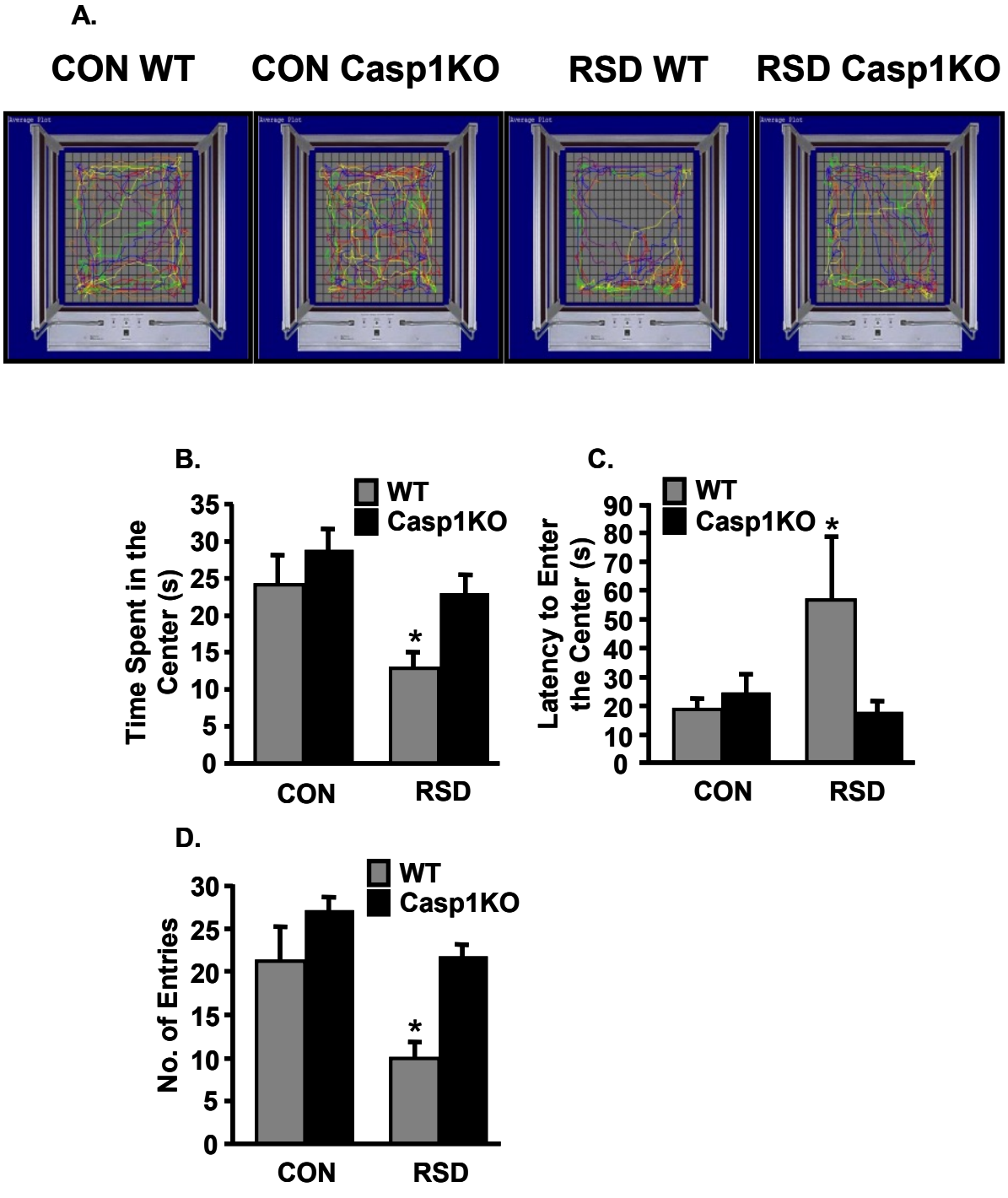
Microglia
Macrophages
IL-1R1

Figure 3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CON Casp1KO
RSD WT
RSD Casp1KO
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Casp1KO
CON
RSD
C.
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Casp1KO
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D.
No. of Entries
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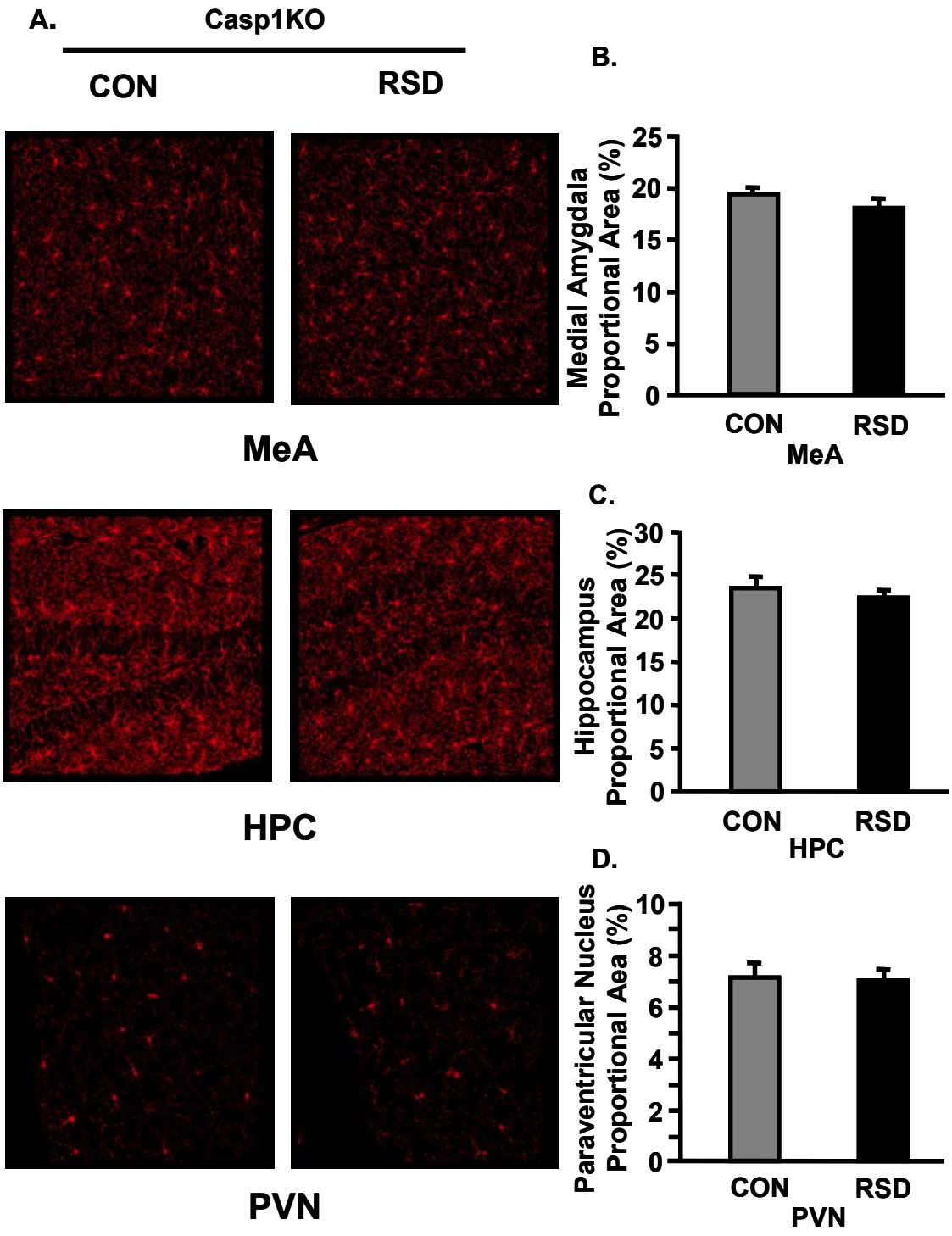
CON
RSD

Figure 4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RSD
MeA
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CON
RSD
MeA
HPC
C.
Hippocampus Proportional Area (%)
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15
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5
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CON
RSD
HPC
PVN
D.
Paraventricular Nucleus Proportional Aea (%)
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8
6
4
2
0
CON
RSD
PVN

Figure 5

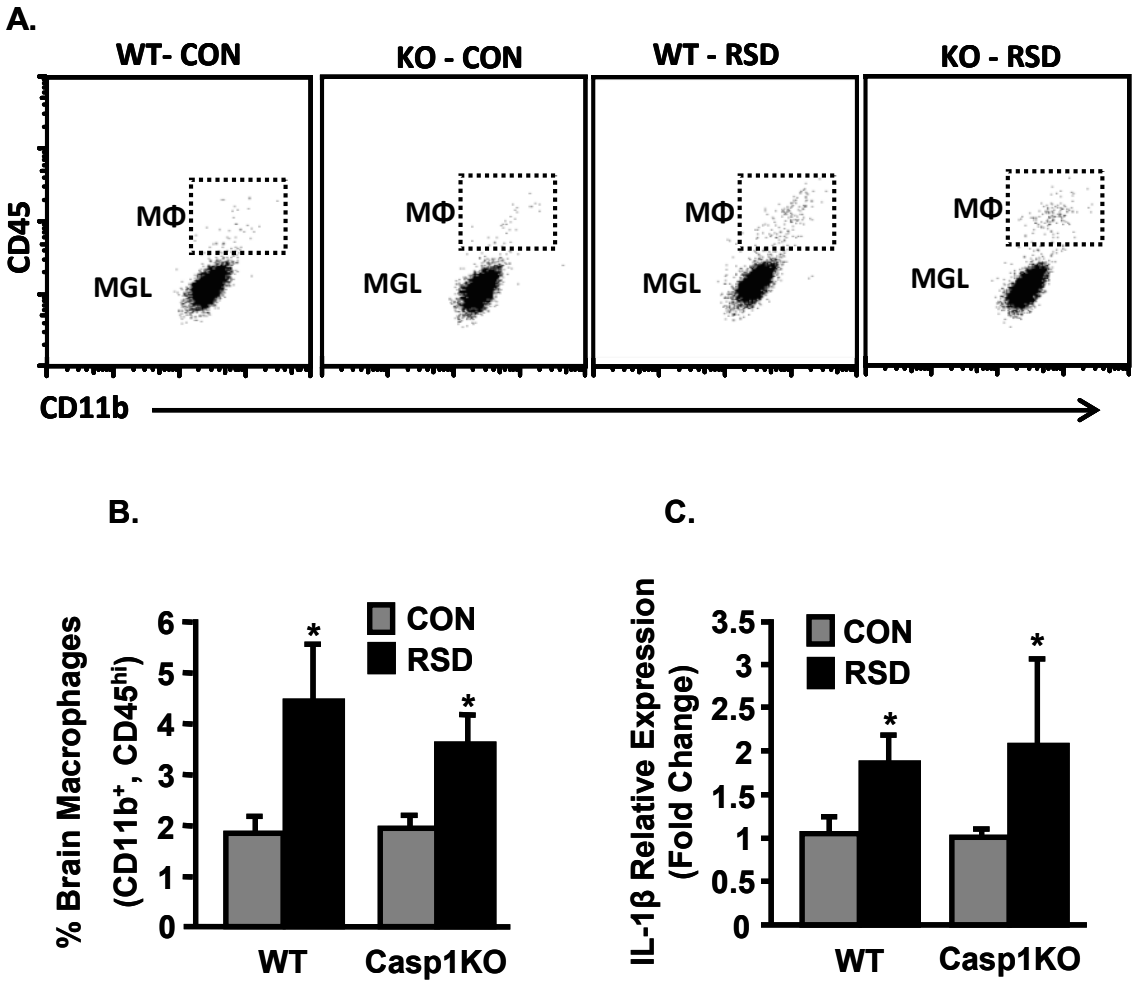
A.
WT- CON
KO - CON
WT - RSD
KO - RSD
CD45
MΦ
MGL
CD11b
B.
% Brain Macrophages
(CD11b+, CD45hi)
CON
RSD
WT
Casp1KO
C.
IL-1β Relative Expression
(Fold Change)
CON
RSD
WT
Casp1KO

Figure Legends

Figure 1: The inflammasome. Cell stress on the innate immune system leads to the activation of TLR and NLR proteins. This induces the NF-κB signaling cascade, leading to the production of pro-IL-1β and pro-IL-18 as well as the activation of NLRP3. NLRP3 recruits intracellular pro-caspase-1 and activates it to become caspase-1. Active caspase-1 then proteolytically cleaves pro-IL-1β and pro-IL-18 to their bioactive and pro-inflammatory forms, IL-1β and IL-18, which is then released from the innate immune cells.

Figure 2: Peripheral macrophages exhibit exaggerated IL-1β expression in the brain following repeated social defeat. C57BL/6 mice were subjected to 6 cycles of social defeat (RSD) or left undisturbed (CON) and were sacrificed 12 hr after the last cycle (n = 3). A) CX3CR1 expression in Microglia and Macrophages. B) LysM expression. C) Ly6C expression. D) CD45 (PTPRC) expression. E) IL-1b expression. F) IL1-R1 expression. Bars represent the mean ± SEM. Bars with different letters (a, b, c) are significantly different from each other.

Figure 3: Caspase-1 deficiency prevented stress-induced anxiety-like behavior. Wild-type (WT) and Caspase-1 Knockout (Casp1KO) C57BL/6 mice were subjected to RSD or CON conditions. Mice were then tested 14 h after the last cycle of stress for anxiety-like behavior in the Open Field test for 5 minutes (n = 7-9, 3 independent experiments). A) Time spent in the center. B) Latency to enter the center. C) Total number of entries into the center. Bars represent the mean ± SEM. Means with asterisk (*) are statistically

significant.

Figure 4: Activated microglial morphology caused by repeated social defeat was prevented by caspase-1 deficiency. Casp1KO mice were subjected to 6 cycles of RSD or CON conditions. Mice were perfused and brains were collected 14 h after the 6th cycle of RSD for Iba-1 staining (n = 2-4). A. Representative images of Casp1KO mice in CON and RSD conditions from the medial amygdala (MeA), dentate gyrus of hippocampus (HPC), and paraventricular nucleus (PVN) are shown (20x). Proportional data for Iba-1 staining in the B) medial amygdala, C) hippcampus, and D) paraventricular nucleus. Increased morphological activation is associated with increased percent area (Wohleb et al. *J Neurosci*, 2011). Bars represent mean ± SEM. Means with asterisk (*) are statistically significant.

Figure 5: Caspase-1 deficiency did not prevent macrophage trafficking to the brain nor increased IL-1β mRNA expression in microglia following RSD. WT and Casp1KO C57BL/6 mice were subjected to RSD or CON conditions. Mice were then sacrificed 14 h after the last cycle of RSD and brains were collected (n = 3). A) Representative bivariate plots for flow cytometry (CD11b, CD45). B) Flow cytometry analysis of percent of brain macrophages (CD11b+, CD45hi) in the brain. C) rt-qPCR analysis of IL-1βfrom microglia (Fold Change). Bars represent the mean ± SEM. Means with asterisk (*) are statistically significant.

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